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Citation for published version:

Viczián, A, Klose, C, Ádám, É & Nagy, F 2017, 'New insights of red light-induced development: Novel molecular aspects of phytochrome B action', *Plant, Cell and Environment*. <https://doi.org/10.1111/pce.12880>

Digital Object Identifier (DOI):

[10.1111/pce.12880](https://doi.org/10.1111/pce.12880)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Peer reviewed version

Published In:

Plant, Cell and Environment

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1 **New insights of red light-induced development**

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22 **Running title:**

23 Novel molecular aspects of phytochrome B action

24

25 **Key words:**

26 phytochrome B, photomorphogenesis, phosphorylation, SUMO, dark reversion

27 **SUMMARY STATEMENT**

28

29 Phytochromes sense changes in the ratio and intensity of R and FR content of sunlight
30 and by initiating/controlling a complex signaling network regulate nearly all aspect of
31 plant growth and development. Recent research revealed exciting new aspects at
32 molecular level how these photoreceptors function, uncovered the basic difference in
33 the mode of action for the two major phytochrome species phyA and phyB and
34 demonstrated that phyB is also function as thermosensor. This review summarizes and
35 discusses the most important discoveries that opened new avenues for phytochrome-B
36 related research

37

38

39 **ABSTRACT (133 words)**

40

41 The red/far-red light absorbing photoreceptors phytochromes regulate development
42 and growth, and thus play an essential role in optimizing adaptation of the sessile
43 plants to the ever changing environment. Our understanding of how absorption of a
44 red/far-red photon by phytochromes initiates/modifies diverse physiological responses
45 has been steadily improving. Research performed in the last five years has been
46 especially productive, and led to significant conceptual changes about the mode of
47 action of these photoreceptors. In this review we focus on the phytochrome B
48 photoreceptor, the major phytochrome species active in light-grown plants. We
49 discuss how its light-independent inactivation (termed dark/thermal reversion), post-
50 translational modification, including ubiquitination, phosphorylation, sumoylation as
51 well as heterodimerisation with other phytochrome species modify red-light-
52 controlled physiological responses. Finally we discuss how photobiological properties
53 of phyB enable this photoreceptor to function also as thermosensor.

54

55

56 **INTRODUCTION**

57

58 Light is a key environmental factor affecting almost every aspect of plants'
59 life. It is not only the main source of energy for photosynthesis, but also acts as a
60 developmental clue to harmonize growth with the ambient light environment, a

61 process termed photomorphogenesis. To alter the developmental program active in the
62 dark (skotomorphogenesis) and thereby to ensure proper photomorphogenesis, plants
63 have evolved a battery of photoreceptors. These sensors monitor the light spectrum,
64 selectively absorb photons with different energies and translate light energy into
65 biological signals to modulate the expression of thousands of genes that ultimately
66 culminate in defined physiological responses. The widely used model plant
67 *Arabidopsis thaliana* possesses the following photoreceptors: (i) the UV
68 RESISTANCE LOCUS 8 (UVR8) absorbs ultraviolet B (Jenkins, 2014), (ii) the
69 phototropins (Christie, 2007), the cryptochromes (Yu *et al.*, 2010) and ZEITLUPE
70 type receptors (Kim *et al.*, 2007) are responsible for blue/UV-A perception, and (iii)
71 phytochromes (phy) absorb red (R) and far-red (FR) light (Bae & Choi, 2008;
72 Franklin & Quail, 2010).

73 Phytochromes exist in two interchangeable forms: the Pr form absorbs R light
74 (λ_{\max} =660 nm), whereas the Pfr form absorbs FR light (λ_{\max} =730 nm). Phytochromes
75 are synthesized in the Pr form in dark-grown seedlings, and absorption of a red photon
76 induces conversion of Pr to Pfr, which is the biologically active phy conformer
77 (Rockwell *et al.*, 2006). Pfr is rapidly converted back to Pr by FR light
78 (photoreversion) or, in the absence of light, by dark reversion, also called thermal
79 relaxation, (Mancinelli, 1994). This interconversion property of phytochromes allows
80 these photoreceptors to function as R/FR-dependent molecular switches. The
81 *Arabidopsis* phytochrome gene family contains five genes encoding phyA through
82 phyE (Clack *et al.*, 1994). They are classified according to their stability: the type I is
83 light-labile (phyA), whereas the type II phytochromes are light-stabile (phyB-E).
84 phyA is the dominant phytochrome of dark-grown (etiolated) seedlings, but its
85 amount decreases rapidly upon illumination. Type II phytochromes are the prevalent
86 phytochromes of light-grown plants; among them phyB is the most abundant
87 (Hirschfeld *et al.*, 1998; Sharrock & Clack, 2002). In photobiological terms three
88 modes of action have been identified for phytochromes. Low fluence responses
89 (LFRs) are typical R/FR reversible responses mediated nearly exclusively by type II
90 phytochromes. Very low fluence responses (VLFRs) are triggered by extremely low
91 quantities of light, mediated by phyA and not photoreversible, whereas the high
92 irradiance responses (HIRs) produced by prolonged exposure to high-intensity light
93 can be mediated by phyA or phyB (Nagy & Schafer, 2002).

94

95 **PHYTOCHROME REGULATED PHYSIOLOGICAL RESPONSES**

96

97 In *Arabidopsis*, phyA plays an important role in seedling establishment during the
98 transition from skotomorphogenesis to photomorphogenesis. This and various other
99 aspects of phyA signalling are discussed in the accompanying chapter in this issue.
100 The switch to light-driven development, however, is not exclusively regulated by
101 phyA. For example, regulation of germination and seedling de-etiolation (Su *et al.*,
102 2015) is mediated, beside phyA (Shinomura *et al.*, 1996), also by phyB and other type
103 II phytochromes (Hennig *et al.*, 2002; Dechaine *et al.*, 2009; Lee *et al.*, 2012; Jiang *et al.*
104 *et al.*, 2016). The latter process results in the spectacular change of seedling morphology
105 and manifests itself as inhibition of hypocotyl elongation, inducing opening of the
106 cotyledon hook and expansion of the cotyledons (McNellis & Deng, 1995; Franklin &
107 Quail, 2010; Kami *et al.*, 2010). In a light-dominated environment the action of type
108 II phytochromes regulates production of functional photosynthetic apparatus,
109 promotes chloroplast development (Chen *et al.*, 2010) alters photorespiration
110 (Igamberdiev *et al.*, 2014), contributes to stomata development (Casson &
111 Hetherington, 2014) and regulates stomata opening (Wang *et al.*, 2010). Apart from
112 these responses phytochromes regulate (i) gravitropic orientation of roots and
113 hypocotyls (Kim *et al.*, 2011; Hopkins & Kiss, 2012) and (ii) development of rosette,
114 branching and apical dominance (Finlayson *et al.*, 2010; Franklin & Quail, 2010),
115 thus, in principle, define the architecture of adult plants (Figure 1A).
116 Pr and Pfr forms of phytochromes have overlapping absorption spectra, thus these
117 photoreceptors are also able to monitor the R/FR ratio of sunlight. This is of particular
118 importance in natural habitats, when light is reflected or filtered through the leaves of
119 neighbouring plants. Under a dense canopy the R/FR ratio of sunlight can
120 dramatically change, because chlorophylls and carotenoids efficiently absorb R but
121 not FR light, which results in a low R/FR ratio. Changes in R/FR ratio drastically
122 modulate phytochrome signalling and trigger the so-called shade avoidance syndrome
123 (SAS). This response, characterized by specific morphological changes such as leaf
124 hyponasty, increased apical dominance, elongated petioles and early flowering, is of
125 great importance for plants as it is essential for overgrowing competitors to optimize
126 the efficiency of photosynthesis (Casal, 2012; Casal, 2013; Fraser *et al.*, 2016). SAS
127 is mediated dominantly by phyB, but all members of the phy family are involved in
128 the response, except for phyC (Franklin *et al.*, 2003). As stated above phyB as phyB

129 Pfr primarily mediates plant growth and development in response to changes in R/FR
130 ratios and fluences in the ambient light environment. However, several lines of
131 evidence indicate that phyB is also functioning under FR-HIR conditions when the
132 majority of phyB molecules exist in their inactive Pr conformation. For example, it
133 has been shown that seedlings overexpressing PHYB-GFP show pronounced
134 etiolation phenotypes compared with the wild type counterparts under FR light
135 (Wagner *et al.*, 1996; Casal *et al.*, 2000; Hennig *et al.*, 2001). This response can also
136 be observed without the presence of phyA thus phyB inhibition of phyA function,
137 under these circumstances, is not mediated by the direct interaction of these
138 photoreceptors. More recently, it was also demonstrated that phyB is required for the
139 proper nuclear accumulation of COP1 and SPA1 in FR, indicating that phyB can
140 modulate the intracellular distribution of signaling components required for proper FR
141 signaling (Zheng *et al.*, 2013). However, other factors such availability of nutrients
142 (Short, 1999) also affect this response thus unravelling the precise molecular
143 machinery for phyB action in FR will require further investigations.

144 Phytochromes, especially phyB, have also been shown to play a role in
145 modulating signalling induced by biotic stress (herbivory) (Ballare, 2009), abiotic
146 salinity (Carvalho *et al.*, 2011) and drought stress (Gonzalez *et al.*, 2012) and
147 thermosensing (Franklin *et al.*, 2014; Johansson *et al.*, 2014; Quint *et al.*, 2016). Two
148 recent papers which will be discussed in detail in this review, revealed the molecular
149 mechanism underlying the role of phyB in integrating light and temperature induced
150 signalling and established phyB as a *bona fide* thermosensor (Jung *et al.*, 2016; Legris
151 *et al.*, 2016). All above described developmental/growth/stress responses similar to
152 timing of flowering (Valverde *et al.*, 2004; Endo *et al.*, 2013) are also regulated by the
153 circadian clock. A direct link between the action of red light receptors and the
154 circadian clock has been already established. On the one hand all phytochromes,
155 dominantly phyB, mediates transmission of light signals to the core clock mechanism
156 (Devlin & Kay, 2000; Mas *et al.*, 2003; Huang *et al.*, 2016) on the other hand, most of
157 the light-regulated processes are modulated by the clock, illustrating the complex
158 mutual interactions of light and clock signalling pathways (Greenham & McClung,
159 2015) (Figure 1A).

160
161
162

163 **STRUCTURE OF PHYTOCHROMES**

164

165 All phytochromes have similar primary structures. The N-terminal domain of
166 the apoprotein consists of the N-terminal extension (NTE), the PAS (PER-ARNT-
167 SIM), the GAF (cGMP-specific phosphodiesterases, adenylyl cyclases and FhlA) and
168 the PHY (phytochrome) domains (Figure 1B). The GAF domain cradles a linear
169 tetrapyrrole chromophore (phytochromobilin) attached via a thioether bond to a
170 conserved cysteine residue, and provides light sensitivity to the molecule (Nagatani,
171 2010). The C-terminal domain has regulatory functions, required for the dimerisation
172 of the molecule; it contains two PAS domains as well as a motif related to histidine
173 kinases (HKRD) (Nagatani, 2010; Vierstra & Zhang, 2011). Expressing the N-
174 terminal domain of type II phytochromes alone proved that this domain is essential for
175 light perception and signal transduction (Matsushita *et al.*, 2003; Oka *et al.*, 2008;
176 Adam *et al.*, 2013). A recent report revealed the crystal structure of the N-terminal
177 domain of Arabidopsis phyB, and provided additional insights into the conformational
178 change underlying phyB signalling (Burgie *et al.*, 2014). The role of the different
179 domains in mediating the interaction of phyB with signalling partners will be
180 discussed in detail later in this review.

181

182 **MOLECULAR MECHANISMS OF PHYB SIGNALLING**

183

184 Light-induced translocation of phyB Pfr from the cytosol into the nucleus is an early
185 and indispensable step in phyB signalling (Fankhauser & Chen, 2008; Klose *et al.*,
186 2015b). In contrast to phyA, which relies on the transport helper proteins FHY1
187 (FAR-RED ELONGATED HYPOCOTYL 1) and FHL (FHY1-LIKE), the
188 mechanism of the light-dependent nuclear import of phyB is not comprehensively
189 understood. PhyB nuclear import occurs independently of FHY1 and FHL
190 (Hiltbrunner *et al.*, 2006). The C-terminal half of phyB lacking the chromophore
191 binding domain is localized in the nucleus independently of light (Sakamoto &
192 Nagatani, 1996; Matsushita *et al.*, 2003). Further experiments demonstrated that
193 intramolecular interactions between the N-terminal and C-terminal domains of phyB
194 occur preferentially in the Pr form and are weakened in the Pfr form. Based on these
195 observations a molecular mechanism has been proposed, in which the conformational
196 transition from the Pr to the Pfr form unmask the nuclear localization motif in the C-

197 terminal domain to promote light-induced import of the photoreceptor into the nucleus
198 (Chen *et al.*, 2005).

199 A more recent study offered an alternative interpretation of the above-mentioned
200 findings. In a cell-free *in vitro* nuclear import system using isolated nuclei of the
201 green alga *Acetabularia*, Pfeiffer *et al.* reconstituted the nuclear import of phyB only
202 in the presence of transport factors that interact with phyB and carry an NLS (Pfeiffer
203 *et al.*, 2012). Interestingly, neither the full-length nor the N-terminal or C-terminal
204 half of *Arabidopsis* phyB alone was able to accumulate in the *Acetabularia* nuclei,
205 indicating that phyB itself does not contain a functional intrinsic NLS-motif. Addition
206 of PIF3 (PHYTOCHROME INTERACTING FACTOR 3) to the system induced
207 nuclear import of phyB as well as of both phyB fragments. PIF3 was previously
208 shown to interact with both the N- and C-terminal halves of phyB, whereby binding to
209 the N-terminal domain was Pfr-dependent (Ni *et al.*, 1998; Ni *et al.*, 1999). In the
210 *Acetabularia* system PIF3-mediated nuclear import of the C-terminal phyB fragment
211 occurred independently of light, whereas that of the N-terminal fragment was clearly
212 red-light-induced, indicating that the higher affinity of PIF3 to the Pfr-form is the
213 reason for its light-dependent accumulation in the nucleus. The minimal requirements
214 for a protein facilitating the nuclear import of phyB were narrowed down to a
215 combination of a phyB-binding domain and an NLS, implying that any protein that
216 interacts with phyB in a Pfr-specific fashion and contains an NLS could potentially
217 mediate light-induced nuclear phyB import. This was further supported by the
218 observation that nuclear import of phyB *in vivo* was impaired but not completely
219 abolished in a *pifq* mutant lacking 4 of the PIF proteins (*pifq* = *pif1pif3pif4pif5*),
220 which indicates that proteins other than PIFs are involved in the nuclear translocation
221 of phyB (Pfeiffer *et al.*, 2012).

222 In the nucleus phyB controls seedling development by inhibiting two classes
223 of repressors of photomorphogenesis: the COP1 (CONSTITUTIVELY
224 PHOTOMORPHOGENIC1)/ SPA (SUPPRESSOR OF phyA-105) complex and the
225 PHYTOCHROME INTERACTING FACTORS (PIFs). These repressors by acting
226 synergistically promote skotomorphogenesis, but are inhibited by photoactivated
227 phytochromes allowing photomorphogenic development in light. In darkness the E3
228 ubiquitin ligase COP1 forms complexes with members of the SPA (SPA1-SPA4 in
229 *Arabidopsis*) and PIF families and targets positive regulators of photomorphogenic
230 growth for degradation by the proteasome (Xu *et al.*, 2014). Phytochromes inactivate

231 the COP1/SPA/PIF complex leading to exclusion of COP1 from the nucleus, resulting
232 in stabilization of its target proteins (Osterlund & Deng, 1998; Subramanian *et al.*,
233 2004; Pacin *et al.*, 2014) and degradation/inactivation of PIFs (Al-Sady *et al.*, 2006).
234 However, until recently the molecular mechanism underlying COP1/SPA inactivation
235 was not understood. It was demonstrated that phyA Pfr and phyB Pfr interact directly
236 with SPA1, and by reorganizing the COP1/SPA complex they promote
237 photomorphogenic development (Lu *et al.*, 2015; Sheerin *et al.*, 2015). These authors
238 show that photoactivated phyB competes with COP1 for SPA binding, thereby
239 disturbing the direct interaction between COP1 and SPA. Since SPA1 has been shown
240 to enhance the E3 ubiquitin ligase activity of COP1 in the complex (Seo *et al.*, 2003),
241 it is not yet clear whether disruption of the COP1/SPA complex by phyB directly
242 interferes with COP1 function on its target proteins, or rather eliminates the positive
243 effect of SPA1 on COP1 activity. The finding that photoactivated phytochromes
244 disrupt the direct interaction of COP1 and SPA provides a mechanistic model to
245 explain the fast inactivation of the COP1/SPA complex independently of the slow
246 process of COP1 exclusion from the nucleus.

247 Accumulation of phyB Pfr in the nucleus further initiates inactivation and
248 degradation of PIFs that act as negative regulators of photomorphogenesis as well.
249 PIFs are basic helix-loop-helix (bHLH) type transcription factors that regulate gene
250 expression to promote skotomorphogenesis (Duek & Fankhauser, 2005; Leivar *et al.*,
251 2008; Shin *et al.*, 2009). Photoactivated phyB directly interacts with PIFs and induces
252 their phosphorylation, ubiquitination and subsequent degradation by the proteasome
253 (Al-Sady, *et al.*, 2006; Shen *et al.*, 2007; Shen *et al.*, 2008; Leivar & Quail, 2011; Ni
254 *et al.*, 2013). Recently, the *in vivo* phosphorylation sites of PIF3 have been determined
255 during dark-to-light transition. Introducing multiple missense point mutations at the
256 phosphorylation sites stabilized the protein in light, whereas phospho-mimic
257 mutations promoted PIF3 degradation in the absence of light. These findings
258 supported the conclusion that light-induced phosphorylation of PIF3 is indeed
259 required for its subsequent degradation and for the negative feedback modulation of
260 phyB levels by PIFs in prolonged light (Ni *et al.*, 2013)

261 Recently Park *et al.* presented evidence that PIF degradation might not be the
262 primary mechanism by which phytochromes inhibit these repressors of
263 photomorphogenesis. The authors showed that the Pfr form of phyB was able to
264 inhibit the DNA binding capacity of PIF3, thereby preventing association to its target

265 promoters *in vivo* (Park *et al.*, 2012). These data indicated that phyB inhibition of PIF
266 function requires interaction of these proteins but mediated by two different
267 mechanisms, i.e. sequestration of PIFs and/or stimulation of their degradation. In this
268 aspect we note that a recent work showed that phyB signalling in one cell, can
269 efficiently initiate PIF degradation in other cells that do not contain phyB. (Kim *et al.*,
270 2016). This observation suggests that phyB initiated cell to cell signalling is involved
271 in controlling activity of PIFs but (i) the chemical identity of the mobile signal(s), (ii)
272 the molecular machinery mediating this type of degradation of PIF3 as well (iii) the
273 overall impact of cell to cell communication on phyB signalling will remain to be
274 elucidated.

275 Based on *in vitro* assays Martinez-Garcia *et al.* have proposed the hypothesis
276 that light-dependent interaction with PIF3 recruits phyB to promoter elements of
277 genomic targets, introducing the idea that phyB could be directly involved in the
278 regulation of gene expression (Martinez-Garcia *et al.*, 2000). On the one hand it has
279 been shown that phyA was able to associate with genomic DNA at promoter elements
280 of numerous genes, many of them were identified as phyA-regulated target gene
281 (Chen *et al.*, 2014). On the other hand a very recent report also demonstrated that
282 phyB, similar to phyA can also be recruited to genomic promoter elements possibly
283 via interaction with DNA-binding transcription factors (Jung *et al.*, 2016). These data
284 indicate that individual and selective modulation of gene expression by phyA and
285 phyB could play an important role in light induced signalling.

286

287 **THE FUNCTIONAL ROLE OF DARK REVERSION IN PHYB SIGNALLING**

288

289 PhyB acts as a light quality and quantity sensor and gradually controls
290 photomorphogenic development depending on the light conditions. Analyses of phyB
291 overexpression lines demonstrated that the light sensitivity of phyB-mediated
292 photomorphogenic responses depends on phyB abundance (Wagner *et al.*, 1991;
293 Rausenberger *et al.*, 2010). More precisely, the number of physiologically active Pfr
294 molecules quantitatively determines the signalling efficiency of phyB. Since the
295 absorption spectra of Pr and Pfr overlap considerably, a dynamic photoequilibrium
296 between the Pfr and the Pr forms is established depending on the wavelength. The Pfr
297 form has a higher energy state than the Pr form and is thermally unstable. Thus
298 relaxation of Pfr into Pr can occur in a light-independent fashion (therefore it is also

299 termed dark reversion), but displays a strong temperature dependency (Schäfer &
300 Schmidt, 1974; Hennig & Schäfer, 2001; Klose *et al.*, 2015a). A fast dark reversion
301 process is able to compete with the light reaction of Pr-to-Pfr formation under non-
302 saturating light conditions, leading to steady state Pfr levels lower than the
303 photoequilibrium (the maximal relative Pfr level established depending on the light
304 quality). Consequently, photoconversion and dark reversion determine the steady state
305 level of the active Pfr conformation, enabling dynamic light quality and quantity
306 sensing.

307 The PAS-GAF-PHY domains of Arabidopsis phyB N-terminal (photosensory
308 module, PSM) recombinantly expressed in *E. coli* and reconstituted with
309 phytochromobilin as chromophore exhibited efficient Pfr-to-Pr thermal reversion *in*
310 *vitro* with a half-life of about 110 min, indicating that dark reversion is a property of
311 the phytochrome molecule (Zhang *et al.*, 2013; Burgie *et al.*, 2014). In contrast, dark
312 reversion of full-length phyB expressed in yeast and reconstituted with
313 phycocyanobilin as chromophore showed very rapid initial dark reversion, but did not
314 revert completely back to Pr (Eichenberg *et al.*, 2000; Sweere *et al.*, 2001). More
315 recent *in vivo* studies, however, revealed that phyB Pfr reverts almost completely to Pr
316 within 4 h of darkness, corresponding to an overall half-life of 60 min (Sweere *et al.*,
317 2001; Rausenberger *et al.*, 2010; Klose *et al.*, 2015a). Taken together, these studies
318 indicate that in addition to being an intrinsic property of the phytochrome molecule,
319 dark reversion is modulated by various external factors as well as intra- and
320 intermolecular interactions.

321 Mutations altering conserved residues surrounding the chromophore in the
322 phyB protein were shown to affect Pfr-to-Pr dark reversion differentially without
323 impairing photoconversion. The Arg352Ala substitution stabilized Pfr against thermal
324 reversion, whereas Arg322Ala caused a substantially faster dark reversion of purified
325 recombinant PSM of phyB *in vitro* (Zhang *et al.*, 2013). Arabidopsis *phyB* mutant
326 seedlings expressing the full-length phyB[Arg352Ala] showed normal phyB
327 signalling under high fluence rates of red light and in white light, but were
328 hypersensitive under low fluence rates, suggesting that thermal reversion impacts
329 phyB action when light conditions are limiting. Consistent with this conclusion, Oka
330 *et al.* showed that the Arg322Gln substitution reduced responsiveness of Arabidopsis
331 seedlings expressing the full-length mutant variant under intermittent red light pulses
332 (Oka *et al.*, 2008).

333 The NTE domain of phyB has been shown to stabilize Pfr, and mutants
334 lacking this domain exhibit accelerated dark reversion *in vitro* (Zhang *et al.*, 2013).
335 The PHY domain contains a unique tongue-like structure that interacts with the GAF
336 domain bearing the chromophore. This protrusion has been implicated in the
337 transmission of conformational changes from the chromophore retained in the GAF
338 domain to the PHY domain and consequently the whole molecule. Thereby the tongue
339 was found to refold during transmission from Pr to Pfr from a beta-strand to an alpha-
340 helix (Takala *et al.*, 2014). Mutations in this tongue region of the PHY domain of
341 phyB, e.g. Arg582Ala, Gly564Glu (*phyB-401*) have been described leading to a
342 dramatically enhanced thermal stability of the Pfr form resulting in strong
343 hypersensitivity of seedlings grown under weak red light (Kretsch *et al.*, 2000; Ádám
344 *et al.*, 2011; Zhang *et al.*, 2013). In addition, the Glu812Lys mutation (*phyB-101*) in
345 the second of the two PAS domains in the C-terminal of phyB (Figure 1B) caused
346 enhanced dark reversion in combination with a loss-of-function phenotype,
347 demonstrating that protein domains that are more distant from the chromophore could
348 also affect Pfr thermal stability (Elich & Chory, 1997). It would be interesting to
349 investigate whether other phyB loss-of-function mutants might be affected in dark
350 reversion as well.

351 Phytochromes form dimers *in vivo*, and dimerization has been shown to be
352 important for their physiological function (Matsushita *et al.*, 2003). Consequently,
353 phytochrome dimers can exist in three different states: Pr-Pr, Pfr-Pr, and Pfr-Pfr. A
354 recent study demonstrated that the different dimer species of phyB indeed exhibit
355 differential kinetic properties that are fundamental for the mode of phyB action (Klose
356 *et al.*, 2015a). Already in 1987 it was proposed that dark reversion has different
357 kinetics for Pfr-Pfr and Pfr-Pr dimers based on *in vivo* observations (Brockmann *et al.*
358 *et al.*, 1987). This was supported by the finding that recombinant Pfr-Pr dimers
359 expressed in yeast showed fast and complete dark reversion in contrast to Pfr-Pfr
360 dimers that remained more stable (Hennig & Schäfer, 2001). Klose *et al.* (2015a)
361 combined *in vivo* measurements and mathematical modelling to demonstrate that Pfr-
362 Pr heterodimers and Pfr-Pfr homodimers exhibit extremely different dark reversion
363 kinetics, with Pfr-Pr dark reversion being almost 100-fold faster as compared to Pfr-
364 Pfr. These findings lead to the conclusion that in *Arabidopsis* the phyB Pfr-Pr
365 heterodimer pool undergoes fast dark reversion, resulting in reduced amounts of
366 active phyB, particularly under light conditions that favour the generation of Pfr-Pr

367 heterodimers, e.g. lower light intensities or wavelengths above 690 nm. As the
368 physiological phyB function is inhibited under such light conditions, it was concluded
369 that only the Pfr-Pfr homodimers in the nucleus are able to initiate phyB-mediated
370 light signalling (Klose *et al.*, 2015a). In other words, the slow dark reversion of the
371 Pfr-Pfr homodimer determines the persistence of phyB signalling after transfer to
372 darkness, whereas the extremely fast dark reversion of the Pfr-Pr heterodimer
373 competes efficiently with the Pr to Pfr photoconversion, reducing the Pfr levels under
374 non-saturating irradiation.

375 The precise nature of the fast Pfr-Pr dark reversion process needs to be
376 determined. It is possible that the thermal stability of the Pfr-Pr dimer is affected
377 when only one of the two subunits has undergone the conformational change from Pr
378 to Pfr. Alternatively, the Pfr form of phyB could be stabilized by interactions with
379 other proteins, for example ARABIDOPSIS RESPONSE REGULATOR 4 (ARR4),
380 and such stabilization may work more efficiently for the Pfr-Pfr homodimer (Sweere
381 *et al.*, 2001). Phosphorylation of specific amino acids, especially that of Ser86
382 residing in the N-terminal domain of phyB can also modify dark reversion and red
383 light signalling by an ARR4-independent mechanism (Medzihradzsky *et al.*, 2013);
384 this is discussed in more detail in the following section.

385 Upon light irradiation, phyB associates within discrete subnuclear structures named
386 photobodies (PBs) (Chen *et al.*, 2003; Fankhauser & Chen, 2008). Light conditions
387 establishing high Pfr levels promote the formation of large PBs *in vivo* (Trupkin *et al.*,
388 2014; van Buskirk *et al.*, 2014). Thus it has been proposed that these PBs function in
389 stabilizing phyB Pfr, which allows phyB to continue controlling the level of PIFs and
390 suppressing hypocotyl growth after light-dark transfer (Rausenberger *et al.*, 2010; van
391 Buskirk *et al.*, 2014; Klose *et al.*, 2015a). Very recently it was shown that PCH1
392 (PHOTOPERIODIC CONTROL OF HYPOCOTYL 1), a protein that is associated
393 with the Evening Complex in Arabidopsis, binds phyB in a red-light-dependent
394 manner and co-localizes with phyB into PBs (Huang *et al.*, 2016). With the need to be
395 verified experimentally, the authors presented a model, in which binding of PCH1 to
396 phyB after light exposure slows dark reversion of phyB Pfr, thereby extending the
397 lifetime of phyB-containing large PBs (Huang *et al.*, 2016). A correlation between
398 dark reversion rates, PB formation and stability has been observed previously: mutant
399 phyB molecules exhibiting accelerated dark reversion often failed to localize to PBs
400 under normal light conditions or required higher fluence rates of red light, whereas

401 mutants with slower dark reversion accumulated into PBs even under weak fluence
402 rates (Ádám *et al.*, 2011; Medzihradzsky *et al.*, 2013; Zhang *et al.*, 2013).

403

404 **POST-TRANSLATIONAL MODIFICATIONS OF PHYB**

405

406 **Ubiquitination**

407 The E3 ubiquitin ligase COP1 was shown to interact with the N-terminal fragment of
408 phyB, it was capable to ubiquitinate the photoreceptor and ubiquitination of phyB was
409 stimulated by the presence of PIF3 in these *in vitro* assays (Jang *et al.*, 2010). More
410 recently, mass-spectrometry analysis of proteins co-purified with PIF3 from
411 Arabidopsis identified components of a Bric-a-Brack/Tramtrack/Broad (BTB)-
412 Cullin3-type E3 ubiquitin ligase as red-light-specific PIF3-interacting proteins (Ni *et al.*
413 *et al.*, 2014). Interestingly, the two highly conserved BTB proteins LRB1 (Light-
414 Response-BTB1) and LRB2 had been previously shown to be required for
415 proteasomal phyB degradation (Christians *et al.*, 2012) Ni *et al.*, however, could show
416 that PIF3 phosphorylation triggers recruitment of LRB E3 ubiquitin ligases to the
417 PIF3-phyB complex, whereupon LRBs promote polyubiquitination and degradation of
418 both PIF3 and phyB *in vivo* (Ni *et al.*, 2014). The proposed PIF3-phyB co-degradation
419 model provides a mechanistic explanation for phyB-induced PIF3 degradation and
420 concurrent signal attenuation by photoreceptor degradation (Zhu & Huq, 2014). PIF3
421 degradation is about 50-fold faster as compared to phyB degradation. The strongly
422 different degradation kinetics of PIF3 and phyB were explained by the different
423 protein levels in seedlings, where phyB is much more abundant than PIF3, which was
424 supported by the fact that overexpression of PIF3 enhanced phyB degradation (Ni *et al.*
425 *et al.*, 2013; Ni *et al.*, 2014). Whereas phyB degradation in red light was completely
426 abolished in an *lrb123* triple mutant, PIF3 degradation was only slowed down. The
427 results are compatible with the hypersensitive phenotype of *lrb123* in light (Christians
428 *et al.*, 2012) that is consistent with the observed higher phyB abundance in light, but
429 not with a defective PIF3 degradation (Ni *et al.*, 2014). These observations suggest
430 that the main function of LRBs is signal attenuation by photoreceptor degradation,
431 and that there is partial functional redundancy between the LRBs and other unknown
432 E3 ligases for PIF3 degradation.

433 **Phosphorylation**

434 Early studies performed using purified oat and maize phytochromes indicated that
435 phytochromes have autophosphorylation activity whereas sequence comparison
436 showed that the C-terminal domain of phytochromes contains a region homologous to
437 bacterial histidine kinases (Schneider-Poetsch *et al.*, 1991). Research performed to
438 clarify how and to what extent (reversible) phosphorylation modulates phyA action
439 produced plenty of data (Kim *et al.*, 2004; Ryu *et al.*, 2005; Han *et al.*, 2010), yet until
440 very recently the significance of the postulated kinase activity of phyA (Yeh &
441 Lagarias, 1998; Fankhauser *et al.*, 1999) was debated (for details see accompanying
442 review article in this issue). Here we only note that a very recent report identified the
443 kinase domains of various plant phytochrome species including oat and Arabidopsis
444 phyA, and demonstrated that this region is critical for ATP-binding (Shin *et al.*,
445 2016). These authors also provided convincing evidence that perturbation of this
446 region inhibited phosphorylation of PIF3 by oat phyA *in vitro*, and confirmed in
447 transgenic plants that the kinase activity of phyA is critical for efficient light-induced
448 signalling.

449 In contrast to phyA, our knowledge about the phosphorylation of phyB is
450 rather limited, although it was shown that (i) PAPP5 and PAPP2c
451 (PHYTOCHROME-ASSOCIATED PROTEIN PHOSPHATASE) proteins bind to the
452 Pfr form of phyB, (ii) their null mutants show reduced responses in R light, and that
453 (iii) phyB is phosphorylated *in vitro* and also interacts with the protein phosphatase
454 PAPPC2 (Ryu *et al.*, 2005; Phee *et al.*, 2008). These observations suggested that
455 phosphorylation of the photoreceptor attenuates light signalling. More recent studies
456 identified a number of phosphorylated residues of phyB (Medzihradzky *et al.*, 2013;
457 Nito *et al.*, 2013). Medzihradzky *et al.* demonstrated that the Ser86 located in the N-
458 terminal domain of the protein is phosphorylated *in planta*. The phospho-mimic
459 phyB[Ser86Asp] mutant shows fast dark reversion, and thereby decreases the amount
460 of phyB Pfr. The low Pfr level of the mutant phyB slows down the import of the
461 receptor into the nucleus and limits its interaction with PIF3; in other words,
462 phosphorylation of phyB effectively attenuates light signalling. Consistent with this
463 conclusion the non-phosphorylatable phyB[Ser86Ala] mutant displays slower dark
464 reversion *in vitro* and *in planta*, thus transgenic plants expressing this mutant exhibit
465 hyperactive responses including inhibition of hypocotyl elongation, cotyledon
466 expansion, shade avoidance and flowering, particularly under low light intensity
467 conditions, where Pfr amount is limiting (Medzihradzky *et al.*, 2013; Hajdu *et al.*,

468 2015). Besides Ser86, work performed by Nito *et al.* revealed nine further
469 phosphorylated amino acid positions in Arabidopsis phyB (Ser84, Tyr89, Tyr90,
470 Tyr91, Ser94, Ser95, Tyr104, Ser106, Tyr113). These amino acids are located in a
471 cluster named PCSM motif (Phosphorylation Cluster of Signaling Modulation)
472 spanning from Ser84 to Tyr113 (Figure 1B) and are conserved evolutionarily,
473 indicating their general regulatory importance (Nito *et al.*, 2013). The phosphorylation
474 of each identified amino acid negatively regulates phyB signalling, but among them
475 Tyr104 has the most pronounced phenotype. Tyr104 is phosphorylated after light
476 exposure, and the phospho-mimic mutant phyB[Tyr104Glu] possesses no light
477 signalling activity at all, whereas the non-phosphorylated phyB[Tyr104Phe] shows
478 enhanced activity as compared to wild-type phyB (Nito *et al.*, 2013). Similarly to
479 Ser86, phosphorylation of Tyr104 also attenuates phyB signalling, presumably also by
480 accelerating dark reversion. These data suggest that this domain of the molecule could
481 be a “hot-spot”, where Pfr stability is regulated according to the actual light
482 conditions.

483 Beside the PCSM domain, phyB was reported to be autophosphorylated at unknown
484 sites within its NTE domain (1-100) by (Phee *et al.*, 2008) *in vitro* and at the Ser596,
485 Tyr601, Ser977, Ser1163 residues *in planta* (Nito *et al.*, 2013). These latter amino
486 acids were phosphorylated in the dark and in the light as well, and the function of
487 these modifications is not known (Nito *et al.*, 2013). A very recent study
488 demonstrated that phyB and phyD – similarly to phyA – have kinase activity,
489 autophosphorylate and can phosphorylate PIF3 *in vitro*. The amino acids critical for
490 ATP-binding reside in the N-terminal domain of phyA (1-651) (Shin *et al.*, 2016).
491 The equivalent N-terminal domain of phyB appears to play a significant role in
492 regulating dark reversion (see dark reversion chapter above). Thus we speculate,
493 although the ATP-binding site and kinase activity of phyB is yet to be identified *in*
494 *planta*, that modulation of dark reversion by reversible autophosphorylation and/or
495 phosphorylation of phyB by other kinases as well its ability to phosphorylate other
496 proteins must be harmonized.

497 **SUMOylation**

498 Reversible, covalent conjugation of Small Ubiquitin-Like Modifier (SUMO)
499 molecules to target proteins regulates protein activity and different cellular responses
500 in eukaryotic cells. The conjugation and removal of SUMO is performed by a small
501 set of enzymes, which have conserved structure throughout different organisms

502 (Miura & Hasegawa, 2010; Hickey *et al.*, 2012; Novatchkova *et al.*, 2012). The
503 sumoylation state of the protein pool depends on various factors (including stress,
504 developmental state, hormonal signalling etc.), furthermore numerous plant SUMO
505 substrates were identified in the past few years (Elrouby & Coupland, 2010; Miller *et*
506 *al.*, 2010).

507 Recently it was reported that phyB is sumoylated *in planta*, the SUMOylated form of
508 phyB accumulates to high levels when the receptor is in the Pfr form, and phyB
509 SUMOylation is reversible (Sadanandom *et al.*, 2015). It was also demonstrated that
510 the target lysine of SUMO conjugation is located in the C-terminal domain of phyB.
511 The sumoylation of the mutant phyB[Lys996Arg] is negligible, and the transgenic
512 plants expressing this receptor are hypersensitive in R light. This phenotype could be -
513 at least partly - explained by the reduced binding of the SUMOylated phyB to the
514 negative regulator transcription factor PIF5. Thus these authors concluded that
515 SUMOylation of phyB attenuates light signalling by reducing the formation/stability
516 of the phyB-PIF complexes (Sadanandom *et al.*, 2015). Consistent with its
517 reversibility, the SUMOylation level of the phyB pool appears to be regulated at least
518 partly by the concerted action of OVERLY TOLERANT TO SALT (OTS) 1 and 2
519 SUMO proteases. OTS1 binds directly to phyB and removes the SUMO from the
520 protein. Compared to wild-type plants, the accumulation level of the SUMOylated
521 phyB pool is higher in the *ots1ots2* mutant plants, which show a hyposensitive
522 photomorphogenic phenotype in R light (Sadanandom *et al.*, 2015). It remains to be
523 seen if SUMOylation – similarly to phosphorylation – also targets, beside phyB, other
524 phytochrome species and/or down-stream signalling components.

525

526 **HETERODIMERIZATION OF TYPE II PHYTOCHROMES**

527

528 For many years, after discovering that phyA purified from dark-grown oat seedlings
529 exists primarily as dimer (Lagarias & Mercurio, 1985) it was generally agreed that the
530 type II phytochromes are also active as homodimers. However, two seminal papers
531 (Sharrock & Clack, 2004; Clack *et al.*, 2009) changed this view. First, these authors
532 demonstrated that Arabidopsis contains multiple species of both homodimeric and
533 heterodimeric phyB and phyD phytochromes, but phyA is present only as a
534 homodimer and does not form heterodimers with any other phytochrome species.
535 Next, they reported that phyC and phyE do not homodimerize, but heterodimerize

536 with phyB and phyD and that the expression/activity of phyC in a *phyBphyD* mutant,
537 where none of its dimerization partners was present, dropped dramatically (Clack *et*
538 *al.*, 2009). Clack *et al.* also showed that not only phyB but phyC and phyD,
539 presumably as members of phyB/phyC and phyB/phyD heterodimers co-
540 immunoprecipitate from seedling extracts with the PIF3 transcription factor in a
541 R/FR-reversible manner (Clack *et al.*, 2009). Although direct interaction of phyC,
542 phyD and phyE with PIF3 has not yet been detected *in planta*, these results show that
543 all phytochromes in homo- or heterodimeric forms appear to function through PIF-
544 mediated pathways.

545 Two more recent reports demonstrated that (i) homodimers of the N-terminal
546 fragments of all type II phytochromes were biologically active in the modulation of R-
547 light-regulated photomorphogenesis (Adam *et al.*, 2013) and that (ii) heterodimers of
548 the N-terminal domains of phyB/phyC, phyB/phyD, phyB/phyC, phyB/phyE etc.
549 generated by using a synthetic biological approach showed slightly different
550 phenotypic responses when compared phyB/phyB. For example, the phyB/
551 phyB[Cys357Thr] heterodimer containing the chromophore-less version of phyB was
552 active in petioles and cotyledons, but not in hypocotyls (Liu & Sharrock, 2013).
553 Taken together, the above findings suggested that the formation of such type II
554 heteromeric photoreceptors increases the potential complexity of R/FR light sensing,
555 for example phyC might signal only as heterodimer, yet the question of how and to
556 what extent remained to be answered. Just recently by using a bottom-up assembly of
557 phytochrome network Sanches-Lamas *et a.*, provided more insight into the biological
558 function of phytochrome heterodimerisation (Sanchez-Lamas *et al.*, 2016). In this
559 elegant study the authors first expressed each of the five phytochromes in the
560 quintuple *phyAphyBphyCphyDphyE* mutant and then created lines expressing pairwise
561 these phy genes in all possible combination. Analysis of this set of mutant plants
562 revealed many new features of the phytochrome network and demonstrated among
563 others that phyB alone is sufficient to confer full hypocotyl, germination responses to
564 R and repress flowering but phyB and phyC co-action is needed to confer
565 responsiveness to photoperiod. These findings indicate that phyB/phyB homodimers
566 are mediating responses to light quality whereas phyB/phyC heterodimers are
567 essential for the manifestation of a proper photoperiodic response. These authors also
568 showed that association of phyB to nuclear bodies also modified by phyC and
569 concluded that phyB/phyC heterodimers are probably active for longer periods in

570 darkness which could be an important factor to repress flowering and hypocotyl
571 elongation especially under short-day conditions. In addition, on the one hand they
572 also clarified individual contribution of phyD and phyE to a variety of light controlled
573 responses, for example they showed that phyE strongly repressed flowering but had
574 little effect on controlling hypocotyl growth. On the other hand they also uncovered
575 synergistic and antagonistic effects of phytochromes in controlling germination and
576 flowering and hypothesized that at least part of these responses is mediated by
577 heterodimers of the various phytochrome species. More importantly they have
578 suggested by analysing a large number transgenic lines expressing these
579 phytochromes at different level that the role of the individual phytochrome species is
580 determined by the intrinsic properties of these photoreceptors (such as ability to
581 heterodimerize, photochemical features, interaction with signaling partners etc.) rather
582 than by the expression level or patterns. Notwithstanding these very convincing data,
583 however, it is also true that even a slight reduction of the phyB expression level
584 significantly alters red light responsiveness, indicating that modification of the ratio of
585 phyB/phyB homodimers by other type II phytochromes could be an important factor.
586 At present, the molecular mechanism regulating/limiting homodimerization and/or
587 heterodimerization of phyB with other type II phytochromes is not known, nor is it
588 known how these phyB-containing heterodimers function, i.e. whether they regulate
589 the expression of genes at least partly different from those regulated by homodimers.
590 Given the importance of dark reversion and post-translational modifications of phyB
591 in regulating red light-induced signalling, we speculate that these could also be
592 affected by heterodimerization with phyC, phyD and phyE.

593

594 **ROLE OF PHYB IN TEMPERATURE SENSING/ INTEGRATION OF LIGHT** 595 **AND TEMPERATURE SIGNALING**

596

597 A growing amount of findings has led to the recognition that light and temperature
598 signals are integrated by multiple mechanisms (Franklin *et al.*, 2014; Johansson *et al.*,
599 2014; Quint *et al.*, 2016). The morphological changes induced by high ambient
600 temperature, collectively summarized as thermomorphogenesis, include the promotion
601 of elongation growth which parallels the response to unfavourable light conditions in
602 vegetational shade (Casal, 2012). Interestingly, PIF4, a positive regulator of the shade
603 avoidance response, was identified as central component of ambient temperature

604 signalling (Koini *et al.*, 2009). PIF4 functions in regulating phytohormone
605 biosynthesis and signalling. Expression of *PIF4* is controlled by the circadian clock
606 through repression by the Evening Complex but is increased by high temperature
607 (Nozue *et al.*, 2007; Nusinow *et al.*, 2011). On the posttranslational level PIF4 activity
608 and abundance is controlled by phyB. PIF4 interacts specifically with light activated
609 phyB leading to its phosphorylation and subsequent degradation (Lorrain *et al.*, 2008).
610 Two very recent complementary studies have demonstrated that phyB directly
611 participates in temperature perception based on the temperature dependency of its
612 kinetic properties (Jung *et al.*, 2016; Legris *et al.*, 2016). Although it has been
613 described previously that dark reversion is strongly temperature dependent (Schäfer &
614 Schmidt, 1974; Hennig & Schafer, 2001; Klose *et al.*, 2015a) the two papers
615 highlighted the role of dark reversion in plant temperature responses considering also
616 the differential properties of the phyB dimers.

617 Jung *et al.* (2016) showed that high temperature accelerates the phyB Pfr decay during
618 night time which is based on the temperature sensitivity of the slow dark reversion
619 process of the Pfr-Pfr homodimer. Active phyB was shown to associate in a
620 temperature dependent manner with promoters of genes that are also targeted by PIFs.
621 Faster phyB dark reversion at higher temperature correlated with the loss of phyB
622 occupancy at target gene promoters leading to the conclusion that phyB could
623 transmit temperature information by inhibiting PIF activity through direct binding at
624 target promoters. These findings were supported by extensive gene expression
625 analyses showing that the warm temperature transcriptome is specifically affected by
626 phytochrome activity during nighttime. Phytochrome null mutants displayed a
627 constitutive warm temperature transcriptome even at low temperatures whereas in the
628 constitutively active phyB[Tyr276His] allele the warm temperature transcriptome was
629 constitutively repressed during night.

630 Legris *et al.* (2016) showed that temperature regulation of phyB Pfr levels is effective
631 not only at night but also during the day. In light, the steady state levels of phyB Pfr
632 are determined by the photoconversion rates, depending on the light quality and
633 intensity, as well as by the fast dark reversion rate of the Pfr-Pr heterodimer (Klose *et al.*
634 *et al.*, 2015a). Using both, *in vitro* and *in vivo* spectroscopic assays, the authors
635 demonstrated that the fast Pfr-Pr dark reversion rate of phyB is strongly sensitive to
636 temperature (Legris *et al.*, 2016). This is particularly obvious under low light
637 conditions, where Pr to Pfr photoconversion is slower. Under such conditions the Pfr-

638 Pr heterodimers are more abundant compared to higher light intensities and might
639 undergo dark reversion rather than absorbing another photon to become Pfr-Pfr. High
640 temperature favors the dark reversion reaction thereby reducing the Pfr steady state
641 levels especially at low light conditions. PhyB containing nuclear bodies reflect the
642 status of phyB since they are mainly composed of Pfr-Pfr homodimers. As a proxy for
643 temperature effects on Pfr-Pfr levels Legris *et al.* (2016) quantified the nuclear body
644 sizes of wild-type phyB and two phyB mutant alleles with suppressed thermal
645 reversion (phyB[Tyr361Phe] and phyB[Arg582Ala]) (Zhang *et al.*, 2013) that are not
646 sensitive to temperature changes for a range of different temperatures and light
647 condition. Although they could not detect a straight correlation between temperature
648 and nuclear body size for wild-type phyB, they observed a strong reduction in nuclear
649 body size at temperatures higher than 20°C. By using a mathematical model
650 describing the relation between Pfr-Pfr levels and nuclear body size they could show
651 independently of the spectroscopic measurements that high temperatures decrease the
652 apparent phyB Pfr-Pfr amount. Mathematical modeling of growth responses mediated
653 by phyB, temperature and phyB-independent pathways further revealed that phyB-
654 mediated temperature effects contribute significantly to growth regulation thereby
655 showing largest effects at low irradiances (Legris *et al.*, 2016).
656 Taken together, these studies support the idea that phyB is physiologically responsive
657 to perceive light and temperature signals at the same time indicating that phyB, in its
658 active Pfr conformation, should also be defined as a temperature sensor.

659

660

661 **CONCLUDING REMARKS**

662

663 Phytochrome signalling is an extensively studied field of photobiology. After learning
664 the basics of the receptors' photochemistry, we have greatly extended our knowledge
665 about the molecular mechanisms of phytochrome action, with a special respect to the
666 identification of phytochrome-interacting protein partners. More recent findings
667 revealed the molecular machinery that mediates integration of phytochrome signalling
668 not only with hormone-induced actions (de Lucas & Prat, 2014; de Wit *et al.*, 2016),
669 but also those induced by various biotic and abiotic stresses (Ballare, 2014; Cortes *et al.*
670 *et al.*, 2016) and by temperature (Jung *et al.*, 2016; Legris *et al.*, 2016). It is predictable

671 that this trend will continue; however this review demonstrates that we still have a lot
672 to learn about the phytochrome photoreceptors themselves.

673

674 **ACKNOWLEDGMENTS**

675 We sincerely apologize to all those colleagues whose important work is not cited
676 because of space considerations.

677 The work in Szeged, Hungary was supported by two Hungarian Scientific Research
678 Fund grants (OTKA, K-108559 and NN-110636) and grants from the
679 Gazdaságfejlesztési és Innovációs Operatív Program (GINOP-2.3.2-15-2016-00015
680 and GINOP-2.3.2-15-2016-00001) to F.N.. Work in Edinburgh, UK was supported by
681 a BBSRC grant BB/K006975/1 to F.N.

682

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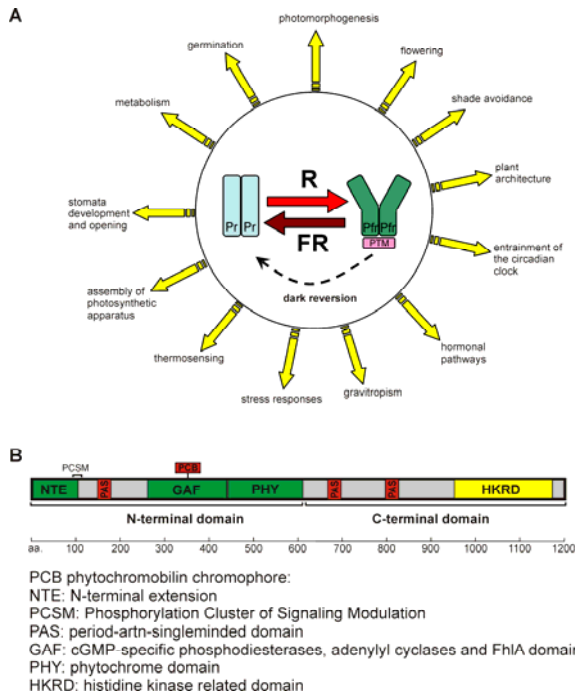
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1087

1088 **FIGURE**

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1092 **Figure 1**

1093 A. Phytochrome B-controlled responses in *Arabidopsis thaliana*.

1094 The ratio of available Pr and Pfr forms of phyB molecules are tuned by the intensity
1095 of red (R) and far-red (FR) light (photoconversion) together with the dark reversion.

1096 The Pr/Pfr dimers are not depicted to maintain clarity (see text for details). PTM
1097 indicates post-translational modifications of the Pfr form.

1098 B. Schematic structure of the phyB monomer.